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Feedback Control of Adrenal Steroidogenesis via H_2O_2-Dependent, Reversible Inactivation of Peroxiredoxin III in Mitochondria

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SUMMARY

Certain members of the peroxiredoxin (Prx) family undergo inactivation through hyperoxidation of the catalytic cysteine to sulfinic acid during catalysis and are reactivated by sulfiredoxin; however, the physiological significance of this reversible regulatory process is unclear. We now show that PrxIII in mouse adrenal cortex is inactivated by H_2O_2 produced by cytochrome P450 enzymes during corticosterone production stimulated by adrenocorticotropic hormone. Inactivation of PrxIII triggers a sequence of events including accumulation of H_2O_2, activation of p38 mitogen-activated protein kinase, suppression of steroidogenic acute regulatory protein synthesis, and inhibition of steroidogenesis. Interestingly, levels of inactivated PrxIII, activated p38, and sulfiredoxin display circadian oscillations. Steroidogenic tissue-specific ablation of sulfiredoxin in mice resulted in the persistent accumulation of inactive PrxIII and suppression of the adrenal circadian rhythm of corticosterone production. The coupling of CYP11B1 activity to PrxIII inactivation provides a feedback regulatory mechanism for steroidogenesis that functions independently of the hypothalamic-pituitary-adrenal axis.

INTRODUCTION

Peroxiredoxins (Prxs) catalyze the reduction of H_2O_2 to water, with a conserved cysteine residue serving as the site of oxidation by H_2O_2 (Rhee and Woo, 2011). Mammalian 2-Cys Prx enzymes (Prx I–IV), a subgroup of Prx family, are unique in that the catalytic Cys undergoes hyperoxidation during catalysis to cysteine sulfinic acid (Cys-SO_2H), resulting in inactivation of peroxidase function (Woo et al., 2003; Yang et al., 2002). Sulfinic 2-Cys Prxs are reduced back to the active form by sulfiredoxin (Srx) in a process that consumes ATP and cellular thiols (Biteau et al., 2003; Chang et al., 2004). The physiological relevance of these processes has, however, remained unknown.

To gain insight into the role of the reversible hyperoxidation of 2-Cys Prx under physiological conditions, we examined various mouse tissues for the presence of sulfinic 2-Cys Prx. PrxIII, a mitochondrion-specific enzyme, was detected in the sulfinic form in the adrenal gland of mice maintained under normal conditions, and sulfinic forms of other Prxs were not detected (see Figure 1). The adrenal cortex is the main site for the production of glucocorticoids (corticosterone [CS] in rodents and cortisol in humans), which is induced by the pituitary hormone adrenocorticotropic hormone (ACTH) in response to stress. Psychological or physical stress induces the release of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) from the hypothalamus. CRH and AVP are transported to the pituitary gland, where they act synergistically to stimulate the secretion of ACTH. ACTH is then transported to the cortex of the adrenal gland, where it rapidly stimulates the biosynthesis and secretion of glucocorticoids. The hypothalamus, pituitary gland, and adrenal gland constitute the HPA axis, a major component of the neuroendocrine system that controls responses to stress. The HPA axis also provides a mechanism for feedback regulation, in which glucocorticoids inhibit their own release by blocking the synthesis of ACTH and CRH in the pituitary gland and hypothalamus, respectively. Administration of the synthetic glucocorticoid dexamethasone (DEX) thus enhances such negative feedback, resulting in a reduction in ACTH secretion (Jefcoate, 2002; Rosol et al., 2001).

ACTH stimulates CS synthesis predominantly through activation of cAMP signaling (Jefcoate et al., 2011; Manna et al., 2009), although signaling pathways involving Ca^{2+} and MAPKs also mediate ACTH action (Manna and Stocco, 2011). The synthesis of CS from cholesterol requires the sequential actions of cytochrome P450 (CYP) enzymes. The rate-limiting step in CS synthesis is the transfer of cholesterol from the outer to the inner mitochondrial membrane, where CYP11A1 catalyzes the first side-chain cleavage reaction (Jefcoate, 2002; Rosol et al., 2001). The last step of CS synthesis is catalyzed by CYP11B1 in mitochondria. The cholesterol delivery to the inner mitochondrial membrane is mediated mostly by steroidogenic acute regulatory protein (StAR) (Miller, 2007). CS synthesis is therefore governed predominantly by mechanisms that determine the synthesis, activation, and degradation of StAR (Jefcoate, 2002; Stocco et al., 2005). StAR is initially synthesized as a 37 kDa protein and is then converted to a 30 kDa form in

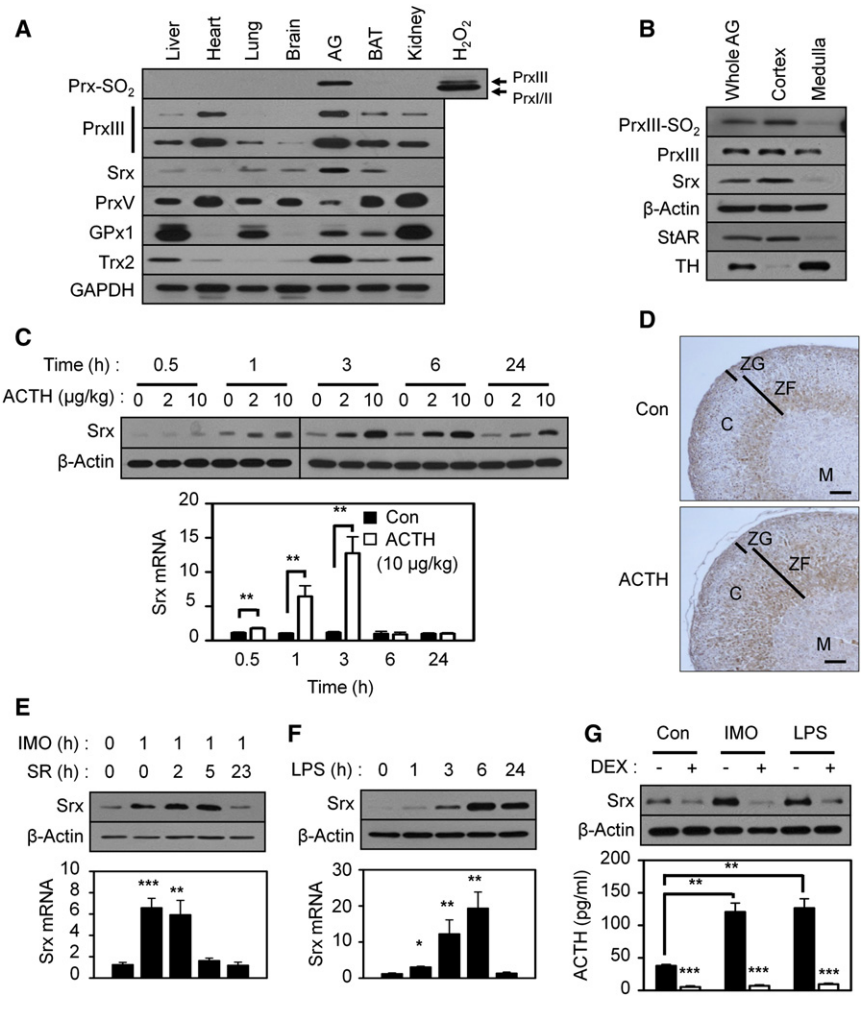

Figure 1. Detection of Sulfinic PrxIII and Srx in Mouse Tissues and ACTH-Dependent Upregulation of Srx in the Adrenal Gland

(A) Homogenates of various mouse tissues were subjected to immunoblot analysis with antibodies to the indicated proteins. A lysate of Y-1 cells that had been exposed to 500 μM H_2O_2 for 10 min was used as a positive control (H_2O_2) for sulfinic PrxI/II and sulfinic PrxIII (rightmost lane). Two different blot exposures are shown for PrxIII. AG, adrenal gland; BAT, brown adipose tissue.

(B) Homogenates of whole adrenal gland (AG), adrenal cortex, or adrenal medulla of mice were subjected to immunoblot analysis with antibodies to the indicated proteins. StAR and tyrosine hydroxylase (TH) were examined as markers for the cortex and medulla, respectively.

(C) Mice were injected with ACTH (2 or 10 μg/kg, i.p.) or saline (0 μg/kg or Con), and at the indicated times thereafter adrenal gland homogenates were prepared and subjected to immunoblot analysis of Srx (upper panel), and the relative amount of Srx mRNA in the adrenal gland was measured by RT and real-time PCR analysis (lower panel). The mRNA data are means ± SD (n = 4). **p < 0.01.

(D) Six hours after injection of mice with saline (Con) or ACTH (10 μg/kg, i.p.), the adrenal glands were removed and subjected to immunohistochemical analysis with antibodies to Srx. C, cortex; M, medulla; ZG, zona glomerulosa; ZF, zona fasciculata. Scale bars, 100 μm.

(E) Mice were exposed to immobilization (IMO) stress for 0 or 1 hr and killed after release from stress (SR) for the indicated times. Adrenal gland homogenates were then subjected to immunoblot analysis of Srx, and the relative amount of Srx mRNA was measured by RT and real-time PCR analysis. The mRNA data are means ± SD (n = 4). **p < 0.01, ***p < 0.001 versus no stress.

(F) Mice were killed at the indicated times after injection with LPS (1 mg/kg, i.p.), and adrenal gland homogenates were subjected to immunoblot analysis of Srx. The relative amount of Srx mRNA in the adrenal gland was also determined by RT and real-time PCR analysis as means ± SD (n = 6). *p < 0.05, **p < 0.01 versus time 0.

(G) One hour after injection of saline or dexamethasone (5 mg/kg, i.p.), mice were left undisturbed (Con), exposed to immobilization stress for 1 hr, or injected with LPS (1 mg/kg, i.p.). The animals were killed 5 hr later, adrenal gland homogenates were subjected to immunoblot analysis of Srx, and the plasma ACTH concentration was measured. ACTH levels are means ± SD (n = 6). **p < 0.01 for the indicated comparisons, ***p < 0.001 versus the corresponding saline value. See also Figure S1.

mitochondria, and it is fully activated by phosphorylation on Ser194 mediated by protein kinase A (Manna et al., 2009; Miller, 2007). The precise path of cholesterol molecules into mitochondria and the regulation of StAR function remain unclear, however (Miller, 2007). The plasma CS level increases rapidly (within several minutes) on stimulation of the adrenal cortex with ACTH. This immediate response is dependent on cAMP, is achieved by the action of pre-existing StAR molecules, and does not involve new protein synthesis (Ariyoshi et al., 1998). In addition to the acute response, CS production is also characterized by a longer-term response to ACTH, which involves maintenance of optimal levels of steroidogenic proteins including StAR via cAMP-dependent regulation of the transcriptional and translational machineries (Jefcoate et al., 2011; Manna et al., 2009).

Oxidative stress results in downregulation of steroidogenesis, with this effect being related to a decrease in the abundance of StAR and the activation of p38 mitogen-activated protein kinase (MAPK) by reactive oxygen species (ROS) (Abidi et al., 2008; Diemer et al., 2003). Steroidogenesis itself induces oxidative stress because electron transfer by CYPs from the donor system (NADPH, adrenodoxin reductase, and adrenodoxin) to the substrate is not perfectly coupled and is therefore leaky. Such leaked electrons react with O_2 to produce superoxide and consequently H_2O_2, as is the case with electrons leaked from the mitochondrial respiratory chain (Hanukoglu, 2006). About 40% of the total electron flow from NADPH was found to be directed to ROS production during steroid hydroxylation by CYP11B1, whereas much smaller amounts of ROS are produced by the CYP11A1 system (Hanukoglu, 2006).

CS synthesis, especially the last step catalyzed by CYP11B1, thus functions as a substantial source of ROS in adrenocortical cells.

We now show that PrxIII is the most abundant mitochondrial antioxidant enzyme in the adrenal gland and that the seeming imperfections of H_2O_2 production by CYP11B1 and reversible inactivation of PrxIII by its own substrate represent an evolutionary adaptation for feedback inhibition of steroidogenesis. Importantly, the levels of inactivated PrxIII, activated p38, and sulfiredoxin undergo circadian oscillations. Transgenic mice with steroidogenic tissue-specific ablation of sulfiredoxin display persistent accumulation of inactive PrxIII and suppression of the adrenal circadian rhythm of corticosterone production.

RESULTS

Detection of Sulfinic PrxIII and ACTH-Dependent Induction of Srx in the Adrenal Gland of Mice

To determine whether the sulfinic form of 2-Cys Prxs is generated under physiological conditions, we first subjected various mouse tissue homogenates to immunoblot analysis with antibodies to this form of 2-Cys Prxs (Prx-SO_2). PrxI and PrxII cannot be separated from each other by SDS-polyacrylamide gel electrophoresis (PAGE), whereas the molecular size of PrxIII is sufficiently larger than that of PrxI/II to allow its separation. Sulfinic PrxIII was detected only in the adrenal gland, whereas the sulfinic form of other 2-Cys Prxs was not detected in any of the tissues examined (Figure 1A). PrxIII, Srx, and mitochondrial thioredoxin (Trx2) were also most abundant in the adrenal gland, whereas glutathione peroxidase 1 (GPx1) and PrxV, both of which are present in mitochondria, were expressed at low levels in the adrenal gland compared with other tissues (Figure 1A). When the amounts of tissue homogenates analyzed were adjusted to contain similar amounts of PrxIII, sulfinic PrxIII was still apparent only in the adrenal gland (Figure S1A). The adrenal gland consists of two distinct parts, the cortex and medulla, which produce steroid hormones and catecholamines, respectively. Although PrxIII was detected in both the cortex and medulla, sulfinic PrxIII, as well as Srx, was apparent only in the cortex (Figure 1B), suggesting that the reversible hyperoxidation of PrxIII may play a role in CS synthesis. By comparison with immunoblot intensities of the sulfinic form of recombinant PrxIII, the extent of PrxIII hyperoxidation in the adrenal cortex of mice (killed between 1000 and 1100 hr) was estimated to be 10%–20% of total PrxIII (Figure S1B). Given that hyperoxidation of 2-Cys Prxs occurs only during the catalytic cycle (Yang et al., 2002), these results suggested that the amount of H_2O_2 produced in the adrenal gland under normal physiological conditions is sufficient to maintain the abundant PrxIII continuously engaged in H_2O_2 reduction and that the level of mitochondrial Srx is not enough to fully counteract the hyperoxidation of PrxIII. The amounts of PrxIII, PrxV, and GPx1 in mitochondria of the adrenal cortex were estimated to be ~8, ~0.25, and < 0.7 μg per milligram of mitochondrial protein, respectively (Figure S1C).

Srx expression is induced in response to several biological stimuli that elicit ROS production (Bae et al., 2009). We studied the induction of Srx in the adrenal gland of ACTH-injected mice. This induction of Srx protein peaked at 3–6 hr after injection of ACTH at two different doses (2 and 10 μg/kg), with the amount of Srx decreasing slowly thereafter (Figure 1C). The amount of Srx mRNA was also increased by ACTH injection, with this effect being maximal (~10-fold increase) at 3 hr and then rapidly diminishing (Figure 1C). This upregulation of Srx occurred mainly in the zona fasciculata, rather than in the zona glomerulosa, of the adrenal cortex (Figure 1D). Given that ACTH is secreted during physical stress as well as in response to lipopolysaccharide (LPS) (Rosol et al., 2001), we examined the expression of Srx in the adrenal glands of mice that had been exposed to immobilization stress or injected with LPS. Srx was upregulated by immobilization stress for 1 hr, with its abundance peaking between 2 and 5 hr after release from stress; the amount of Srx mRNA was also increased, peaking between 0 and 2 hr after release (Figure 1E). LPS also induced Srx expression, with the abundance of both the mRNA and protein peaking at 6 hr after stimulation (Figure 1F). Administration of DEX before immobilization stress or LPS injection blocked the increase in the circulating concentration of ACTH induced by each treatment (Figure 1G). The induction of Srx by stress or LPS was also blocked by DEX (Figure 1G), suggesting that such induction is mediated by ACTH.

ACTH Increases the Amounts of Sulfinic PrxIII and Phosphorylated p38 MAPK through H_2O_2 Produced by CYP11B1 during CS Synthesis in the Adrenal Gland of Mice

We next monitored the production of CS and sulfinic 2-Cys Prxs in the adrenal gland of mice injected with ACTH. The concentration of CS in plasma reached a maximum within 1 hr (the earliest time point) and had decreased to almost the original value by 6 hr (Figure 2A). The abundance of sulfinic PrxIII in the adrenal gland was increased ~2-fold at 1–3 hr after ACTH injection and decreased thereafter, whereas the total amount of PrxIII did not change (Figure 2B). These data suggested that ACTH-induced steroidogenesis is accompanied by increased H_2O_2 production in mitochondria, which results in the hyperoxidation of PrxIII and consequent accumulation of H_2O_2. Given that H_2O_2 activates p38 MAPK in adrenal gland and hypoxic cardiomyocytes (Abidi et al., 2008; Kulisz et al., 2002), we monitored p38 activation by immunoblot analysis of phosphorylated p38 (p-p38). The amount of p-p38 in the adrenal gland was markedly increased at 1–3 hr after ACTH injection, the same time period at which the accumulation of sulfinic PrxIII was maximal (Figure 2B). The concurrent increases in the amounts of sulfinic PrxIII and p-p38 likely reflected overflow of H_2O_2 accumulated in mitochondria into the cytosol. Although Srx expression is upregulated by ACTH (Figures 1C and 2B), ACTH did not substantially affect the expression of antioxidant proteins such as PrxI to PrxVI, SOD1, SOD2, catalase, Trx1, Trx2, and GPx1 or that of steroidogenic proteins such as StAR, CYP11A1, and CYP11B1 in the adrenal gland, with only heme oxygenase-1 (HO-1) showing increased expression at 6 hr after ACTH injection (Figure S2A).

In the experiments with ACTH-injected mice, saline was injected as a control. A saline injection by itself, however, will inevitably induce stress and stimulate the production of ACTH and CS, thereby affecting the levels of PrxIII-SO_2, p-p38, and

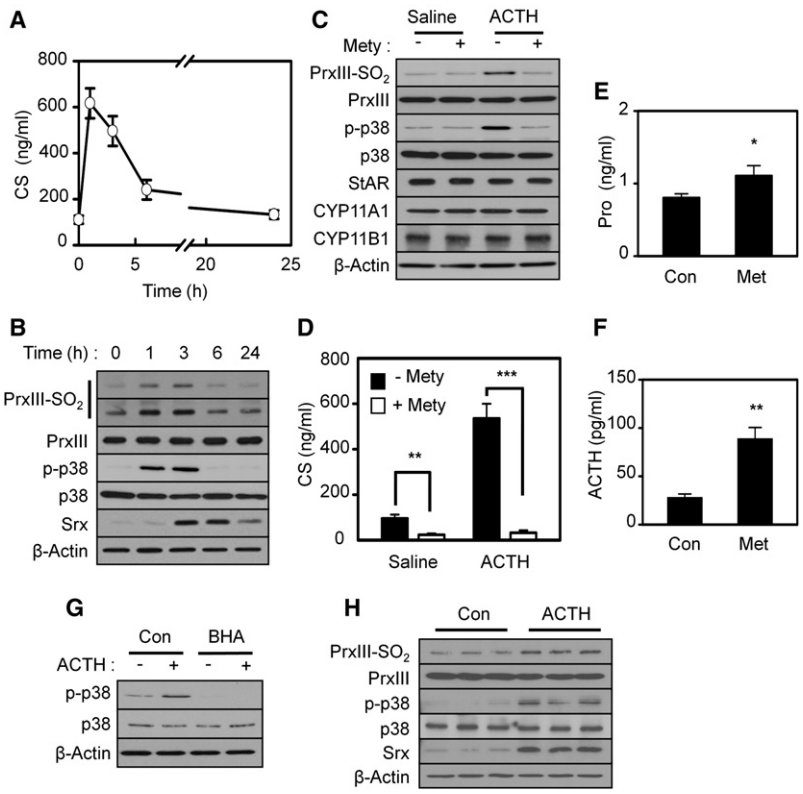

Figure 2. Effects of ACTH on the Levels of PrxIII-SO_2, p-p38 MAPK, and Srx in the Adrenal Gland

(A and B) Mice were injected intraperitoneally (i.p.) with ACTH (10 μg/kg) and killed at the indicated times thereafter. Plasma CS levels (means ± SD, n = 10 to 12) were measured (A), and adrenal gland homogenates were prepared and subjected to immunoblot analysis (B).

(C–F) Mice were injected with saline (–) or metyrapone (Mety; 100 mg/kg, i.p.) and 1 hr later were injected with saline or ACTH (10 μg/kg, i.p.). The animals were killed 3 hr after ACTH administration, adrenal gland homogenates were subjected to immunoblot analysis (C), and the plasma levels of CS (D), progesterone (E), and ACTH (F) were measured. Data are means ± SD (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.

(G) Mouse adrenal glands were incubated in the absence (Con) or presence of 200 μM BHA for 1 hr and then in the additional absence or presence of 500 nM ACTH for 3 hr, after which adrenal gland homogenates were subjected to immunoblot analysis.

(H) Three fresh bovine adrenal glands were obtained from a local slaughterhouse and sliced to isolate the cortex. The cortical slices were incubated in the absence (Con) or presence of 500 nM ACTH (n = 3 each) for 3 hr at 37°C, after which the tissue was homogenized and subjected to immunoblot analysis. See also Figure S2.

Srx in the adrenal gland. To evaluate such placebo effects, we injected mice with DEX to suppress the synthesis of ACTH and CRH before the injection of saline or ACTH. The levels of CS and PrxIII-SO_2 in mice injected with saline alone were clearly higher than those in mice injected with saline after DEX treatment (Figure S2B). The abundance of Srx and p-p38 also appeared to be slightly decreased when mice were treated with DEX prior to saline injection. However, the extent of these effects of DEX, especially those on the levels of Srx, PrxIII-SO_2, and p-p38 MAPK, were relatively small compared with those induced by ACTH (Figure S2B). Placebo injections were therefore routinely performed without prior DEX treatment.

The ACTH-induced increases in the amounts of both sulfinic PrxIII and p-p38 in the adrenal gland were prevented by prior treatment of mice with the CYP11B1 inhibitor metyrapone (Figure 2C). Metyrapone also blocked basal as well as ACTH-induced CS production (Figure 2D), whereas progesterone production, which depends on CYP11A1 but not on CYP11B1, was slightly increased by metyrapone treatment (Figure 2E), suggesting that CYP11A1 activity was not inhibited by metyrapone. The plasma ACTH level was increased in mice injected with metyrapone, likely due to decreased feedback inhibition of ACTH secretion by CS (Figure 2F). The levels of CYP11A1 and CYP11B1 in the adrenal gland were not affected by either ACTH or metyrapone (Figure 2C). These results are consistent with previous data showing that CYP11B1 is the main source of H_2O_2 production during steroidogenesis in the adrenal gland (Hanukoglu, 2006), and they suggested that H_2O_2 produced by CYP11B1 is required for hyperoxidation of PrxIII and for phosphorylation of p38. A role for H_2O_2 in p38 activation was also shown by the observation that prior incubation of the adrenal gland in vitro with the antioxidant butylated hydroxyanisole (BHA) inhibited ACTH-induced p38 phosphorylation (Figure 2G).

The principal glucocorticoid in nonrodent mammals is cortisol, whose synthesis, like that of CS, is also mediated by CYP11A1 and CYP11B1. Stimulation of bovine adrenal glands with ACTH also resulted in PrxIII hyperoxidation, p38 phosphorylation, and Srx induction (Figure 2H).

p38 MAPK Negatively Regulates ACTH-Induced Steroidogenesis by Inhibiting StAR Synthesis

Although the underlying mechanism is not known, activation of p38 MAPK is associated with downregulation of both StAR activity and steroid synthesis in Leydig cells (Diemer et al., 2003). To examine the effect of p38 activation on CS synthesis in the adrenal gland, we incubated the mouse adrenal gland in medium containing the p38 inhibitor SB202190 before stimulation with ACTH. ACTH-induced CS production was increased by ~50% by prior exposure of the adrenal gland to SB202190 (Figure 3A). The basal level of CS production was also enhanced by the p38 inhibitor. In addition, SB202190 potentiated the ACTH-induced hyperoxidation of PrxIII, as would be expected from the increased H_2O_2 production accompanying CS synthesis (Figure 3B). However, neither ACTH nor SB202190 had a measurable effect on the amount of StAR protein (Figure 3B). Regulation of StAR activity is a complex process that involves multiple signaling pathways that coordinate the

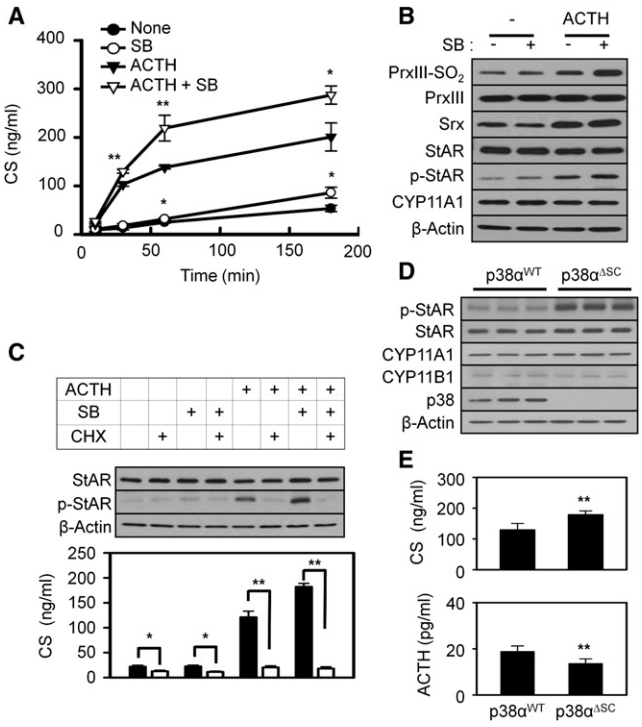

Figure 3. Relation between p38 MAPK Activity and StAR Expression

(A and B) Mouse adrenal glands were incubated in the absence or presence of 10 μM SB202190 (SB) for 1 hr and then in the additional absence or presence of 500 nM ACTH for the indicated times, after which the concentration of CS in the medium was measured (A). Immediately after the last time point for CS measurement in (A), adrenal gland homogenates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins (B). CS data are means ± SD (n = 6). *p < 0.05, **p < 0.01 versus control (None).

(C) Mouse adrenal glands were incubated with or without cycloheximide (CHX, 0.1 mg/ml) or 10 μM SB202190 (SB) for 1 hr and then in the additional absence or presence of 500 nM ACTH for 1 hr. Adrenal gland homogenates were then subjected to immunoblot analysis of StAR and p-StAR, and the concentration of CS in the culture medium was measured. CS values are means ± SD (n = 6). *p < 0.05, **p < 0.01.

(D) Adrenal gland homogenates prepared from wild-type ($p38\alpha^{WT}$) and $p38\alpha^{\Delta SC}$ mice were subjected to immunoblot analysis with antibodies to the indicated proteins.

(E) Plasma concentrations of CS and ACTH in $p38\alpha^{WT}$ and $p38\alpha^{\Delta SC}$ mice. Data are means ± SD (n = 8). **p < 0.01. See also Figure S3.

transcriptional machinery as well as posttranscriptional and posttranslational mechanisms (Manna et al., 2009). It is generally accepted, however, that cholesterol delivery depends largely on StAR molecules that are newly synthesized and phosphorylated at Ser194 (Artemenko et al., 2001). Consistent with this notion, the amount of phosphorylated StAR (p-StAR) was increased in adrenal glands treated with ACTH and SB202190 (Figure 3B), and the protein synthesis inhibitor cycloheximide completely blocked CS production and phosphorylation of StAR induced by ACTH in the absence or presence of SB202190 (Figure 3C). However, none of these agents affected StAR abundance (Figure 3C), likely because the amount of newly synthesized StAR is much smaller than that of the inactive protein present within mitochondria of the adrenal gland.

The effects of SB202190 on StAR expression and steroidogenesis were also evaluated in Y-1 adrenocortical cells, which express only a low level of StAR and produce progesterone in response to intracellular cAMP accumulation. Treatment of the cells with SB202190, forskolin (an activator of adenylyl cyclase), or both agents increased the amount of StAR (Figure S2A). The same treatments also increased the production of progesterone by the cells, and the extent of steroid production appeared to correlate with the amount of StAR in the cells, with the effect of SB202190 plus forskolin being the largest (Figure S2A). These results suggested that, like the cAMP signaling pathway, the p38 signaling pathway regulates steroidogenesis by modulating the abundance of StAR. When Y-1 cells were supplied with 22R-hydroxycholesterol, which is able to permeate the inner mitochondrial membrane independently of StAR, treatment with SB202190 increased StAR abundance but did not affect progesterone production (Figure S2A). SB202190 also did not increase StAR levels in Y-1 cells in the presence of cycloheximide (Figure S2B), suggesting that p38 inhibits new StAR synthesis.

Among the four p38 isoforms (α, β, γ, and δ), SB202190 selectively inhibits the α and β isoforms, and p38α is highly expressed in adrenocortical cells (Abidi et al., 2008; Kulisz et al., 2002). To further study the role of p38 in StAR synthesis, we generated a mouse model ($p38\alpha^{\Delta SC}$) in which p38α is ablated specifically in steroidogenic tissues. Expression of p38 was not detected in the adrenal gland of $p38\alpha^{\Delta SC}$ mice but was unaffected in the liver (Figure S2C). The ablation of p38 had no effect on the abundance of StAR protein, CYP11A1, and CYP11B1, but markedly increased the level of p-StAR (Figure 3D), supporting the notion that p38 negatively regulates new StAR synthesis. Plasma CS levels were increased by ~40% in $p38\alpha^{\Delta SC}$ mice compared with those in $p38\alpha^{WT}$ mice, whereas the circulating ACTH concentration was reduced by ~30% in $p38\alpha^{\Delta SC}$ mice (Figure 3E). This decreased ACTH level was likely due to increased feedback inhibition of ACTH secretion by CS. Ablation of p38α resulted in only a moderate increase in the plasma CS level, probably because of a continued contribution of other p38 isoforms or unknown compensatory mechanisms.

Ablation of Srx Induces Suppression of Steroidogenesis and Accumulation of Cholesterol in the Adrenal Gland

To gain further insight into the role of Srx in steroidogenesis, we generated a mouse model ($Srx^{\Delta SC}$) in which Srx is ablated specifically in steroidogenic tissues (Figures S4A and S4B). The amounts of Srx mRNA (Figure S4C) and protein (Figure S4D) in the adrenal gland of $Srx^{\Delta SC}$ mice were ~10% of those in the adrenal gland of wild-type (Srx^{WT}) mice, whereas the abundance of Srx protein was unaffected in the brain and was actually increased in brown adipose tissue of the mutant animals (Figure S4D). The reduced abundance of Srx in the zona fasciculata of the adrenal gland of $Srx^{\Delta SC}$ mice was also confirmed by immunohistochemical analysis (Figure S4E).

Plasma CS levels were reduced significantly in $Srx^{\Delta SC}$ mice compared with those in Srx^{WT} mice, whereas the circulating ACTH concentration was increased by ~80% in $Srx^{\Delta SC}$ mice (Figure 4A). The plasma level of norepinephrine was not affected

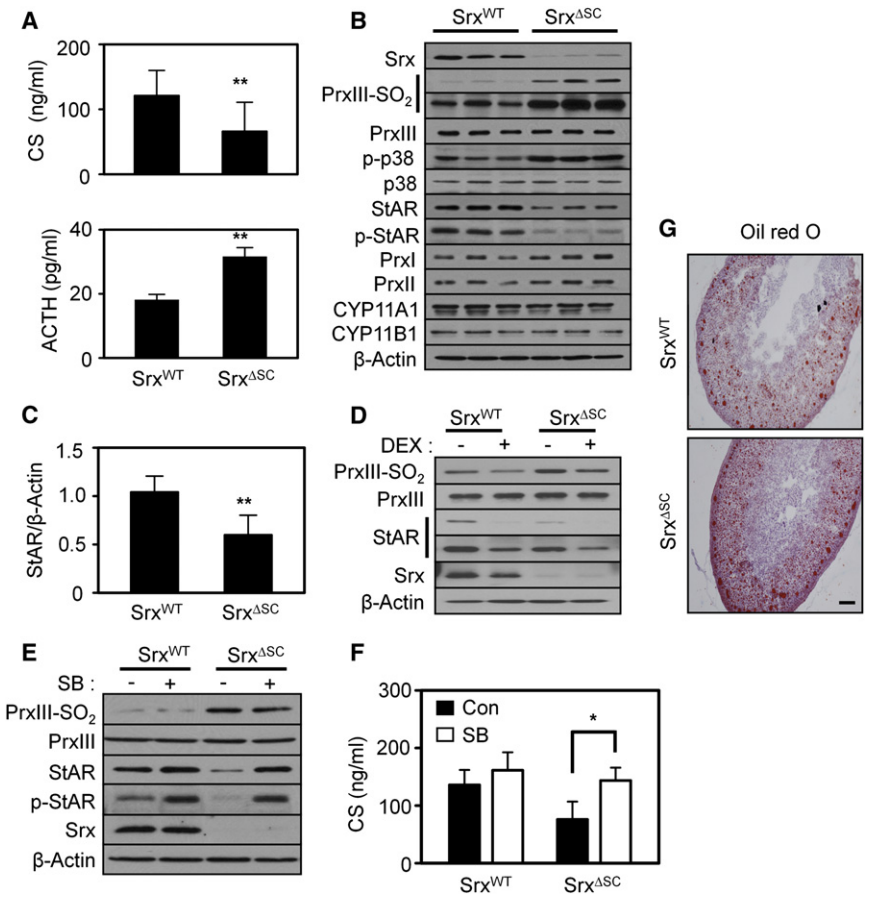

Figure 4. Effects of Steroidogenic Tissue-Specific Deletion of Srx on CS Production and the Levels of Sulfinic PrxIII and StAR in the Mouse Adrenal Gland

(A) CS and ACTH levels in plasma of Srx^{WT} and $Srx^{\Delta SC}$ mice. Data are means ± SD (n = 12). **p < 0.01.

(B and C) Adrenal gland homogenates prepared from Srx^{WT} and $Srx^{\Delta SC}$ mice were subjected to immunoblot analysis with antibodies to the indicated proteins (B). Immunoblot intensities of StAR relative to those of β-actin were also determined (C); data are means ± SD (n = 6). **p < 0.01.

(D) Srx^{WT} and $Srx^{\Delta SC}$ mice were injected with dexamethasone (DEX; 2 mg/kg, i.p.) or vehicle (–). At 6 hr after injection, adrenal glands from the mice were subjected to immunoblot analysis with antibodies to the indicated proteins.

(E and F) Srx^{WT} and $Srx^{\Delta SC}$ mice were injected twice a day for 5 days with vehicle (– or Con) or SB202190 (SB; 0.2 mg/kg, i.p.). At 3 hr after the last injection, adrenal glands from the mice were subjected to immunoblot analysis with antibodies to the indicated proteins (E) and CS levels in plasma were determined (F). CS data are means ± SD (n = 6). *p < 0.05.

(G) Oil red O staining of adrenal glands of Srx^{WT} and $Srx^{\Delta SC}$ mice. Scale bars, 100 μm. See also Figure S4.

in $Srx^{\Delta SC}$ mice (Figure S4F), suggesting that function of the adrenal medulla was not impaired by Srx ablation. Immunoblot analysis revealed that ablation of Srx resulted in a marked increase in the abundance of sulfinic PrxIII as well as an ~40% decrease in that of StAR and a similar decrease in that of p-StAR in the adrenal gland (Figures 4B and 4C). The amounts of PrxI to PrxIII, CYP11A1, and CYP11B1 in the adrenal gland did not differ substantially between Srx^{WT} and $Srx^{\Delta SC}$ mice (Figure 4B). These results suggested that the reduced plasma CS level of $Srx^{\Delta SC}$ mice is due mostly to downregulation of StAR protein rather than to that of other steroidogenic proteins.

Administration of DEX for 6 hr reduced sulfinic PrxIII and StAR in the adrenal gland of $Srx^{\Delta SC}$ as well as Srx^{WT} mice (Figure 4D), indicating that PrxIII hyperoxidation in $Srx^{\Delta SC}$ mice is also mediated by H_2O_2 generated by ACTH-dependent CS synthesis and that the expression and maintenance of StAR in $Srx^{\Delta SC}$ mice are dependent on signaling by ACTH as they are in Srx^{WT} mice. The abundance of StAR and p-StAR in the adrenal gland as well as the plasma concentration of CS in $Srx^{\Delta SC}$ mice increased to levels similar to those in Srx^{WT} mice in response to injection with SB202190 twice a day for 5 days, whereas the amounts of StAR and p-StAR in the adrenal gland of Srx^{WT} mice were also increased slightly by the same treatment (Figures 4E and 4F). These data suggested that the downregulation of StAR apparent in $Srx^{\Delta SC}$ mice is attributable to p38 activation induced by the constitutively increased H_2O_2 levels that result from the inability to reactivate sulfinic PrxIII.

StAR deficiency gives rise to congenital adrenal hyperplasia, in which steroidogenesis is greatly impaired, resulting in cholesterol accumulation in steroidogenic cells (Hasegawa et al., 2000). Histological analysis with oil red O showed that the number and size of vacuoles associated with neutral lipids were increased in the adrenal gland of $Srx^{\Delta SC}$ mice compared with that of Srx^{WT} mice (Figure 4G). In addition, analysis of adrenal gland lipid extracts indicated that the amount of cholesterol esters, a storage form of cholesterol, was increased by ~50% in the adrenal gland of $Srx^{\Delta SC}$ mice (Figure S4G). Microarray analysis also revealed a total of 29 genes whose expression level was increased at least 1.7-fold in the adrenal gland of $Srx^{\Delta SC}$ mice relative to Srx^{WT} mice (data not shown). Among these genes, those for Cidea, Fsp27, and peroxisome proliferator-activated receptor γ (PPARγ) are related to the formation of lipid droplets (Nishino et al., 2008). RT and real-time PCR analysis confirmed that the abundance of Cidea, Fsp27, and PPARγ mRNAs was increased 2- to 3-fold in the adrenal gland of $Srx^{\Delta SC}$ mice (Figure S4H).

The levels of CS, sulfinic PrxIII, and p-p38 in Srx^{WT} and $Srx^{\Delta SC}$ mice were compared after injection with ACTH. The increase in the plasma CS concentration in response to ACTH injection was smaller in $Srx^{\Delta SC}$ mice than in Srx^{WT} mice (Figure 5A). Consistent with the results shown in Figure 2B, the administration of ACTH to Srx^{WT} mice resulted in an increase in the amount of sulfinic PrxIII in the adrenal gland that peaked at 3 hr and

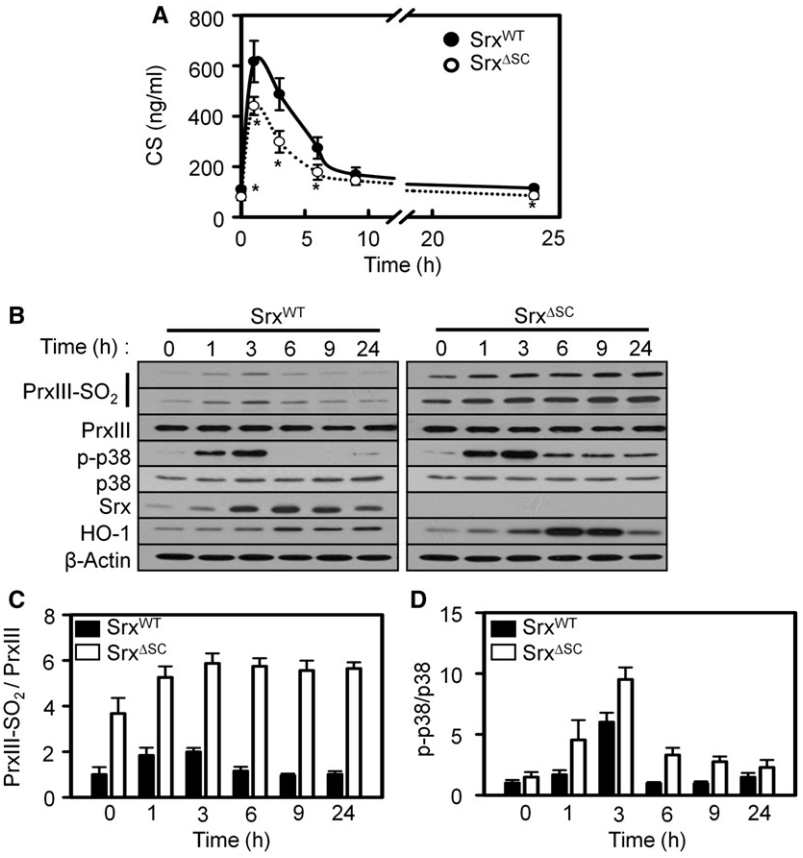

Figure 5. Effects of Steroidogenic Tissue-Specific Srx Ablation on CS Production and the Levels of Sulfinic PrxIII and p-p38 MAPK in the Adrenal Gland of Mice Injected with ACTH

(A) Time course of plasma CS concentration in Srx^{WT} and $Srx^{\Delta SC}$ mice injected with ACTH (10 μg/kg, i.p.). Data are means ± SD (n = 6). *p < 0.05 versus corresponding value for Srx^{WT}.

(B–D) Adrenal gland homogenates prepared from Srx^{WT} and $Srx^{\Delta SC}$ mice at the indicated times after injection of ACTH were subjected to immunoblot analysis (B). The intensities of sulfinic PrxIII bands relative to those of total PrxIII bands (C), and the intensities of sulfinic PrxIII bands relative to those of total p38 bands (D) were estimated from immunoblots similar to those in (B). Data are means ± SD (n = 4). HO-1, heme oxygenase-1. See also Figure S5.

diminished slowly thereafter, as well as an increase in the abundance of p-p38 that also peaked at 3 hr but diminished more rapidly (Figure 5B). As expected, the amount of sulfinic PrxIII in the adrenal gland of ACTH-injected $Srx^{\Delta SC}$ mice increased to levels that were much higher than those apparent in Srx^{WT} mice and remained elevated (Figures 5B and 5C). The abundance of p-p38 in the adrenal gland of ACTH-stimulated $Srx^{\Delta SC}$ mice also peaked at 3 hr, but achieved levels that were much higher than those apparent in Srx^{WT} mice; the amount of p-p38 decreased rapidly thereafter, but remained higher than that in Srx^{WT} mice (Figures 5B and 5D). The finding that the amounts of sulfinic PrxIII and p-p38 increase concurrently in $Srx^{\Delta SC}$ mice as well as in Srx^{WT} mice supported the notion that the accumulation of mitochondrial H_2O_2 as a result of PrxIII inactivation is responsible for p38 phosphorylation. However, the observation that the level of p-p38 decreased rapidly after achieving its ACTH-induced peak in $Srx^{\Delta SC}$ mice, even though the amount of sulfinic PrxIII remained high, suggested that dephosphorylation of p-p38 is determined by factors other than the mitochondrial release of H_2O_2.

The amounts of CS produced by adrenal glands of $Srx^{\Delta SC}$ mice in vitro in the absence or presence of ACTH were also smaller than those produced by adrenal glands of Srx^{WT} mice (Figure S5A). As observed with adrenal glands from ACTH-injected mice (Figure 5B), the levels of sulfinic PrxIII and p-p38 in adrenal glands stimulated with ACTH in vitro were greater for $Srx^{\Delta SC}$ mice than for Srx^{WT} mice (Figure S5B).

Circadian Variation in Sulfinic PrxIII, p-p38 MAPK, StAR, and Srx Abundance in the Adrenal Gland

The levels of ACTH and CS show a daily oscillation, with a peak around the onset of night, in rodents (Oster et al., 2006; Son et al., 2008). We measured the circadian changes in plasma CS concentration at 3 hr intervals during a 12 hr light, 12 hr dark cycle (light on from 0600 to 1800 hr) in Srx^{WT} and $Srx^{\Delta SC}$ mice. Consistent with previous observations, plasma CS levels of Srx^{WT} mice exhibited circadian oscillation during the light-dark cycle (Figure 6A). Immunoblot analysis revealed that the amounts of sulfinic PrxIII, p-p38 MAPK, StAR, and Srx in the adrenal gland of Srx^{WT} mice also showed daily variation, whereas there were no changes in the amounts of PrxIII or p38 (Figures 6B and S6A). The amount of sulfinic PrxIII increased after CS production had peaked, that of p-p38 increased in parallel with the accumulation of sulfinic PrxIII, and that of StAR began to decrease as p-p38 increased. These results are consistent with the notion that H_2O_2 produced during CS synthesis results in the gradual accumulation of sulfinic PrxIII, which in turn leads to further accumulation of H_2O_2 and consequent phosphorylation of p38, which then results in inhibition of nascent StAR production and consequently in that of CS production.

Ablation of Srx resulted in a decrease in plasma CS levels at all time points (Figure 6A). However, although the amplitude was much reduced, the phase of the daily oscillation in CS levels remained unchanged. The abundance of sulfinic PrxIII in the adrenal gland of $Srx^{\Delta SC}$ mice was much higher than that in Srx^{WT} mice, and it showed no circadian variation (Figure 6B). As expected from the increased abundance of sulfinic PrxIII (and the consequent increased levels of H_2O_2), the abundance of p-p38 was higher and that of StAR was lower in the adrenal gland of $Srx^{\Delta SC}$ mice than in Srx^{WT} mice (Figures 6B and S6A). Like the plasma level of CS, the amounts of both p-p38 and StAR in the adrenal gland showed circadian variation, but with a much reduced amplitude in $Srx^{\Delta SC}$ mice (Figures 6B and S6A). These results suggested that PrxIII hyperoxidation is linked not only to the synthesis of CS but also to its circadian

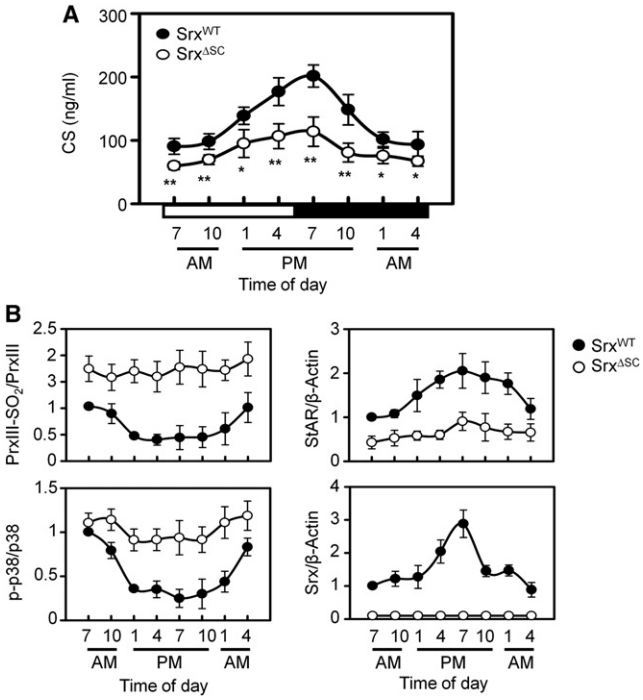

Figure 6. Circadian Variation in Plasma CS as well as Adrenal Sulfinic PrxIII, p-p38 MAPK, StAR, and Srx Levels of Srx^{WT} and $Srx^{\Delta SC}$ Mice

(A) Plasma CS levels in Srx^{WT} and $Srx^{\Delta SC}$ mice were measured at 3 hr intervals during a 12 hr light, 12 hr dark cycle. Data are means ± SD (n = 6). *p < 0.05, **p < 0.01 versus Srx^{WT} mice.

(B) The adrenal glands of mice studied in (A) were subjected to immunoblot analysis with antibodies to sulfinic Prx, to PrxIII, to p-p38 MAPK, to p38, to StAR, to Srx, and to β-actin. The intensities of sulfinic PrxIII and p-p38 bands relative to those of total PrxIII and total p38 bands, respectively, were estimated from immunoblots similar to those in Figure S6. The blot intensities of StAR and Srx were normalized by those of β-actin. Data are means ± SD from six immunoblots (two blots each for three mice of each genotype). See also Figure S6.

variation via the H_2O_2-p38-StAR pathway. At the same time, given that CS production as well as the amounts of p-p38 and StAR still undergo daily variation, albeit with a much reduced amplitude, in the absence of changes in the amount of sulfinic PrxIII in the adrenal gland of $Srx^{\Delta SC}$ mice, factors other than PrxIII hyperoxidation also likely contribute to the circadian variation of CS, p-p38, and StAR.

DISCUSSION

The oxidative inactivation of 2-Cys Prxs has been speculated to be an evolutionary adaptation that allows H_2O_2 to accumulate for a signaling function (Wood et al., 2003). However, sulfinic Prx and the protective effect of Srx have been observed only in cells or tissues exposed to damaging levels of oxidative stress (Bae et al., 2009; Planson et al., 2011). We have now found that 10%–20% of PrxIII exists in the sulfinic form in the mouse adrenal cortex, whereas this form of PrxIII was not detected in other tissues of mice maintained under normal laboratory conditions. The abundance of sulfinic PrxIII in the adrenal gland increased in response to ACTH stimulation as a result of H_2O_2 produced mostly by CYP11B1 during CS synthesis. Given that Prx molecules undergo hyperoxidation only when engaged in the catalytic cycle, the extent of PrxIII hyperoxidation is a measure of how much H_2O_2 is removed by PrxIII (Rhee and Woo, 2011). The accumulation of inactive PrxIII would thus be expected to result in the buildup of H_2O_2 in mitochondria and its overflow into the cytosol.

Various approaches adopted in the present study indicate that such a buildup of mitochondrial H_2O_2 results in the activation of p38 MAPK. An increase in the abundance of sulfinic PrxIII in the adrenal gland was thus always accompanied by a concomitant increase in that of p-p38. Hydrogen peroxide mediates the activation of p38 and JNK by activating ASK1, an upstream kinase of these two stress-activated MAPKs (Matsuzawa and Ichijo, 2008). The inhibition of p38 was previously shown to result in activation of progesterone synthesis, likely through stimulation of StAR expression (Diemer et al., 2003). We found that inhibition of p38 enhanced ACTH-induced CS production, but neither ACTH nor a p38 inhibitor had a measurable effect on StAR abundance in the adrenal gland. Only after depletion of pre-existing StAR protein by exposure of mice to DEX was such an effect of ACTH and p38 inhibition apparent. This is because whereas CS production depends on newly synthesized StAR, steroidogenic cells often contain a large fraction of inactive, intramitochondrial StAR that interferes with detection of the newly synthesized protein (Artemenko et al., 2001; Stocco et al., 2005). During the circadian cycle, however, oscillation of StAR was apparent in the adrenal gland without depletion of inactive StAR, probably because the intramitochondrial protein was actively degraded during the cycle. Furthermore, the increase in the abundance of StAR resulting from p38 inhibition was readily apparent in Y-1 cells, which contain only a small amount of the inactive protein.

StAR regulation has been revealed to be a complex process that is mediated at multiple levels, including the well-established cAMP-dependent transcriptional machinery, posttranscriptional processing and translation of StAR mRNA, and posttranslational modification (Manna et al., 2009). The phosphorylation of StAR on Ser194 by protein kinase A is thought to be required to render the newly synthesized protein fully active with regard to its capacity to support cholesterol transport (Artemenko et al., 2001). In the present study, ACTH and the p38 inhibitor did not substantially affect the level of StAR mRNA in the adrenal gland (data not shown), but they increased the abundance of phosphorylated (newly synthesized) StAR. The reciprocal relationship between p38 and p-StAR could be also seen in the adrenal gland of $p38\alpha^{\Delta SC}$.

We showed that stress signals (ACTH, immobilization, and LPS) induce Srx expression at both the mRNA and protein levels. It has been shown that expression of Srx is upregulated via activator protein-1 (AP-1) or nuclear factor erythroid 2-related factor 2 (Nrf2) (Bae et al., 2009) and that ACTH induces transcriptional activity of AP-1(Beuschlein et al., 2001). It appears that on stimulation of ACTH receptor, Srx is produced via a cAMP-AP-1 pathway (unpublished data) and then translocates into mitochondria in response to oxidative stress

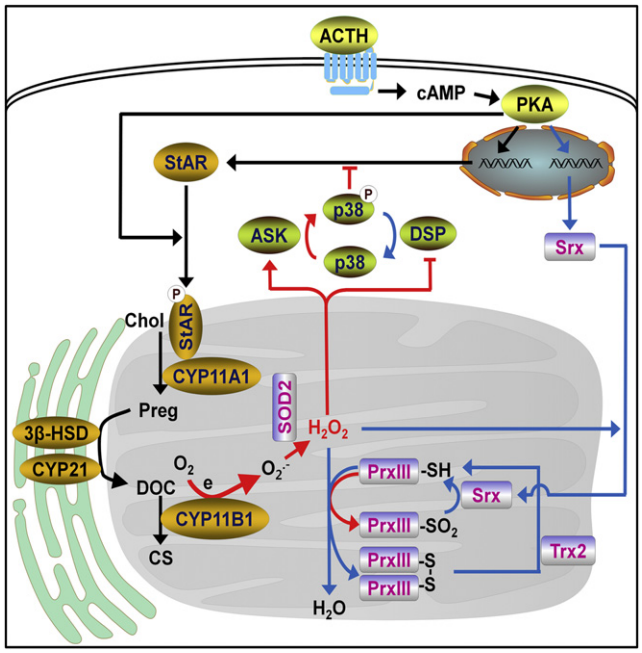

Figure 7. Model for the Role of CYP-Generated H_2O_2, Reversible Hyperoxidation of PrxIII, and Induction of Srx in ACTH-Induced Steroidogenesis in the Adrenal Cortex

The steps leading to CS synthesis are indicated by black arrows, the steps leading to H_2O_2 accumulation and feedback inhibition of StAR synthesis are indicated by red arrows, and the steps leading to a decrease in the concentration of H_2O_2 to basal levels are indicated by blue arrows. See Discussion for details.

(Noh et al., 2009). To study the role of Srx in CS synthesis, we ablated Srx specifically in steroidogenic tissues of mice. Such Srx ablation (~90%) resulted in upregulation of the amount of sulfinic PrxIII in the adrenal gland compared with that in wild-type mice. This increased PrxIII inactivation resulted in increased levels of H_2O_2 and consequent activation of p38 as well as in downregulation of the synthesis and phosphorylation of StAR. CS synthesis was thus much reduced in Srx-deficient mice compared with that in wild-type mice. As a result of the downregulation of CS synthesis in Srx-deficient mice, deposition of cholesterol esters was markedly increased in the adrenal gland.

The daily oscillation of CS is driven via the HPA axis under the control of the master circadian clock, which resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN thus activates the rhythmic release of CRH from the hypothalamus, which evokes circadian ACTH release from the pituitary gland, which in turn regulates the circadian release of CS from the adrenal cortex (Oster et al., 2006). In addition, the SCN clock signal can be transmitted to the adrenal cortex through sympathetic neurons that innervate the adrenal medulla. As the output of the HPA axis, CS is secreted in discrete ultradian pulses, with a period of ~1 hr in the rat (Lightman et al., 2008). In addition to the master clock, the adrenal peripheral clock controls the synthesis of StAR independently of the ACTH circadian rhythm in mice, and the changes in StAR correlate with those in the CS level in blood (Son et al., 2008). In the present study, we observed circadian rhythms of Srx, sulfinic PrxIII, and p-p38 in the adrenal gland, whereas the total amounts of p38 and PrxIII remained unchanged. The opposite oscillations of CS and sulfinic PrxIII, the parallel oscillations of sulfinic PrxIII and p-p38, and the opposite oscillations of p-p38 and StAR observed during the circadian cycle support the link between CS synthesis, hyperoxidation of PrxIII, phosphorylation of p38, and synthesis of StAR. These daily fluctuations are likely important aspects of the peripheral clock specific to the adrenal gland. Ablation of Srx resulted in a decrease in plasma CS levels at all time points during the circadian cycle. In $Srx^{\Delta SC}$ mice, oscillation of sulfinic PrxIII was no longer apparent, whereas CS, p-p38, and StAR continued to oscillate, but with much reduced amplitudes. These results suggest that PrxIII hyperoxidation is linked not only to the synthesis but also to the circadian variation of CS via the H_2O_2-p38-StAR pathway. They also suggest that factors other than PrxIII hyperoxidation contribute to the circadian variation of CS, p-p38, and StAR.

On the basis of our findings, we propose a model for the mechanism underlying the feedback regulation of ACTH-induced steroidogenesis via PrxIII hyperoxidation (Figure 7). Binding of ACTH to its receptor triggers the cAMP-PKA signaling pathway, which results in the rapid phosphorylation and consequent activation of StAR, which mediates the delivery of cholesterol from the outer mitochondrial membrane to CYP11A1 located on the matrix side of the inner mitochondrial membrane. CYP11A1 catalyzes the oxidative cleavage of the cholesterol side chain to produce pregnenolone, which is then converted to progesterone (not shown in Figure 7) by 3β-HSD and then to 11-deoxycorticosterone by CYP21 in the endoplasmic reticulum. CS synthesis is completed at the inner mitochondrial membrane, where CYP11B1 catalyzes the hydroxylation of 11-deoxycorticosterone. In a later stage of ACTH stimulation, the synthesis of steroidogenic proteins including StAR and CYPs is increased in a manner dependent largely on cAMP-PKA signaling in order to meet the increased demand for CS (or to maintain their optimal levels).

The oxidation reactions catalyzed by CYP11A1 and CYP11B1 require donation of electrons from NADPH through the intermediary of mitochondrial AdxR and Adx, which are located on the matrix side of the inner mitochondrial membrane. The process of electron transfer, especially that involving CYP11B1, leaks a large portion (~40% for CYP11B1) of the total electron flow from NADPH, resulting in reduction of O_2 to the superoxide anion ($O_2^{\cdot-}$), which is rapidly converted to H_2O_2 by SOD2. Leakage by the CYP11B1 system increases in the presence of its substrate, 11-deoxycorticosterone. CS production is thus accompanied by generation of H_2O_2. PrxIII is by far the most important H_2O_2-eliminating enzyme in mitochondria of the adrenal cortex. In the H_2O_2-elimination reaction, two reduced PrxIII subunits (PrxIII-SH) are converted to a disulfide-linked dimer (PrxIII-S-S-PrxIII), which is then reduced back to PrxIII-SH by Trx2. During catalysis, PrxIII is occasionally but inevitably hyperoxidized to sulfinic PrxIII (PrxIII-SO_2) and becomes inactivated. This hyperoxidation is reversed by Srx. The fraction of PrxIII molecules that undergo hyperoxidation is low, but proportional to the number of H_2O_2 molecules reduced by the enzyme. In the early

stage of ACTH stimulation, the level of sulfinic PrxIII does not increase substantially above the basal level because the activity of Srx in mitochondria is sufficient to counteract the low level of hyperoxidation. At a later stage of such stimulation, when CS synthesis is increased, H_2O_2 production also increases and is followed by an increase in the level of PrxIII hyperoxidation. The capacity of Srx in mitochondria is now no longer sufficient to counteract this increased hyperoxidation, resulting in the accumulation of inactive PrxIII and the consequent buildup of H_2O_2 in mitochondria and its overflow into the cytosol, where it triggers the phosphorylation of p38 by activating ASK1 and prolongs the lifetime of the phosphorylated state by inactivating dual-specificity phosphatase (DSP). Phosphorylated (activated) p38 inhibits StAR synthesis through an unknown mechanism, resulting in downregulation of the production of CS.

Independently of the H_2O_2 buildup, Srx is synthesized likely as a downstream response to cAMP signaling. In addition, in response to the oxidative stress in mitochondria, Srx translocates into these organelles to catalyze the reduction of PrxIII-SO_2 to active PrxIII-SH, which in turn lowers the level of H_2O_2 to that existing before ACTH stimulation. The timing of Srx translocation relative to that of sulfinic PrxIII accumulation is not clear.

It has been puzzling both why Prxs are inactivated via hyperoxidation of the active site cysteine by their own substrate (H_2O_2) and are reactivated via an ATP-consuming process catalyzed by Srx, as well as why certain CYPs like CYP11B1 waste reducing equivalents and thereby generate toxic oxidants. Our results now indicate that the leakiness of CYP11B1 does not represent a metabolic imperfection, but rather that it evolved together with reversible PrxIII hyperoxidation in order to serve a useful purpose. The extent of PrxIII hyperoxidation is proportional to the number of H_2O_2 molecules removed by PrxIII, which in turn is proportional to the number of CS molecules synthesized. The accumulation of H_2O_2 resulting from the buildup of sulfinic PrxIII thus signals that a sufficient amount of CS has been synthesized. The seeming imperfections of CYP leakiness and Prx hyperoxidation together thus provide a negative feedback mechanism that functions independently of the HPA axis. Given that both the accumulation of hyperoxidized PrxIII and its reduction by Srx are relatively slow, this feedback mechanism may not be consequential in the initial acute phase of CS release or in the pulsatile ultradian release of CS in response to stress, but our data suggest that it is critical for the circadian rhythm of CS production.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Rabbit antisera specific for PrxI–VI, for hyperoxidized 2-Cys Prx, or for Srx were described previously (Chang et al., 2004; Woo et al., 2003). Antibodies to StAR, to CYP11B1, to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or to total p38 MAPK were obtained from Santa Cruz Biotechnology; those to CYP11A1 or to tyrosine hydroxylase were from Millipore; those to GPx1, to catalase, to SOD1, to SOD2, to Trx1, or to Trx2 were from Young In Frontier; those to p38 MAPK phosphorylated on Thr180 and Tyr182 were from Cell Signaling; and those to HO-1 were from Stressgen. Antibodies to β-actin as well as ACTH (1–24), forskolin, metyrapone, LPS, BHA, and DEX were obtained from Sigma. SB202190 were obtained from Calbiochem. Rabbit antibodies to StAR phosphorylated at Ser194 were prepared as described (Jo et al., 2005) and subjected to affinity purification with the use of Sepharose 4B resin (GE Healthcare) conjugated with the corresponding nonphosphorylated form of the antigenic phosphopeptide.

Animals

Male C57BL/6J mice were obtained from the Jackson Laboratory. Conditional Srx KO ($Srx^{\Delta SC}$) mice were obtained by crossing mice with a floxed *Srx* allele with Scc-iCre mice (Figure S4, Supplemental Experimental Procedures). All mice were housed in a temperature-controlled room with a 12 hr light, 12 hr dark cycle, and all experiments were performed with age-matched male mice between 2 and 3 months of age. A week before sacrifice, animals were housed individually. For exposure to immobilization stress, mice were placed in a 50 ml conical tube with the bottom removed. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Ewha Woman's University.

Assay of CS, Progesterone, and ACTH

Plasma was isolated from mouse blood and immediately frozen at −80°C until assay. The concentrations of CS, progesterone, and ACTH in plasma or in culture medium were determined with the use of enzyme-linked immunosorbent assay kits (DRG Diagnostics).

Other Methods

Mitochondria were isolated with the use of a mitochondrial isolation kit (Pierce). Adrenal gland and cell culture are described in Supplemental Experimental Procedures.

Statistical Analysis

Quantitative data are presented as means ± SD and were analyzed with Student's t test. A p value of < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.molcel.2012.05.030.

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